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THE EFFECT OF LOW TEMPERATURE ON THE SPIROCHETES OF RELAPSING FEVER

II. THE STRUCTURE AND MOTILITY OF *SPIROCHAETA NOVI*

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The interesting results obtained in the preservation of the spirochetes of relapsing fever, particularly those findings related to the stage of the disease, indicated that marked changes occurred in the organisms when they were subjected to low temperature. The object of the present study was to determine the variations in the structure and motility of *Spirochaeta novyi* when cooled, frozen, and thawed.

Numerous descriptions of the structure of the spirochetes of relapsing fever are contained in the literature. Two of the early comprehensive studies on *Spirochaeta novyi* were made by Norris, Pappenheimer, and Flournoy (1906) and by Novy and Knapp (1906). The organisms were described as small, thin spirals. The contents of the cells appeared to be homogeneous. Many of the longer forms had a pale-staining band near the center, and in the later stages of the disease irregularly staining organisms were seen. Highly refractive dots in the protoplasm of some of the spirochetes were noted by Breinl and Kinghorn (1906). In some cases the pale-staining periplast was drawn out to form a terminal process. The dark-staining central core was broken up in some of the organisms, revealing a light-staining periplastic sheath. Both ends were pointed, but one end, through the extension of this sheath, was often prolonged into a flagellumlike process from which the central core was absent.

Obermeier (1873) observed the rapid whipping motion of the organisms as they moved among the red cells in the blood specimens from patients with relapsing fever. The most characteristic movement was described by Norris, Pappenheimer, and Flournoy (1906) as "a very active wavy or corkscrew one, broken by an asystolic interval of variable length. This movement may apparently start from either end, and an alternation in the wave of contraction is always noted, except when a spiral becomes attached by one end to some object. A very definite lateral oscillation also takes place. . . . As their vitality becomes impaired, however, their movements grow less rapid, and often assume a tetanic character. When the corkscrew movements become slow, and occur at longer intervals, it can readily be made out that the motion in one direction originates at one extremity, the other extremity determining motion in the reverse direction." The continual reversal of the direction of rotation with the brief quiescent stage between reversals was also observed by Novy and Knapp (1906).

¹ Based on data submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bacteriology, University of Michigan, November, 1944.

They found that the whipping motion of the organisms often persisted for days and even weeks in defibrinated blood stored at room temperature.

EXPERIMENTAL

There is so little variation in structure and motility among the strains of the spirochetes of relapsing fever that differentiation on these bases is difficult. Therefore, *S. novyi*, the American strain, was selected for the following studies.

In order to establish standards for the determination of changes resulting from cooling, freezing, and thawing of spirochetes, observations were made of the normal structure of the organisms at progressive stages of the disease. Three albino rats were inoculated intraperitoneally with equal amounts of a saline suspension of blood infected with *S. novyi*. Four blood films were prepared daily for five days, from the blood obtained from the tail veins of each rat. The films were stained with a modification of Romanowsky's stain and Loeffler's flagella stain. Examination of the films revealed the following: Twenty-four hours after inoculation of the animal there was only an occasional medium or long form which was thick and heavily granulated. In 48 hours there were a few large, granular, and some small, coiled or irregularly spiraled, evenly stained organisms. After 72 hours there were many cells which showed a pale-staining center band; many medium and small, coiled, uniformly stained forms were present. By 96 hours there was considerable clumping and matting together and a predominance of medium and long, granular spirochetes. After 120 hours there were a few granular, fragmented organisms and an occasional thin spiral.

The structure of the spirochetes which had been rapidly frozen and thawed was compared with that of the controls prepared before in order to determine the effects of low temperature on the organisms. Ten rats were inoculated intraperitoneally with equal amounts of a saline suspension of infected rat blood. Each rat was anesthetized and blood was drawn from the heart at 24-hour intervals for 2 to 5 days after inoculation. After defibrination, blood films from each sample were prepared on one-half of each of three slides. The remainder of the blood was transferred to cellophane tubes² and stored at -48°C in a specially constructed "deep-freeze."³ After one week the samples were thawed in a water bath at 37°C and films made on the remaining halves of the slides. Two of the films of each set were stained with Romanowsky's and the third with Loeffler's flagella stain.

A comparison of the films showed that, in general, there was more alteration in the morphology and structure in the frozen and thawed spirochetes which had been obtained late in the disease than at the earlier stages. However, there was considerable variation among the organisms present in any one film. Frozen specimens of the 48-hour period showed a more regular, angular spiral form than the controls. Many of the frozen forms were straightened until the spiral morphology had practically disappeared. Some were shrunken, thin, and

² Obtained from the Lusteroid Container Company, Inc., South Orange, N. J.

³ Manufactured by the Motor Products Corporation, North Chicago, Ill.

distorted with granules and swellings. There appeared to be a slight decrease in the number of organisms present after freezing and thawing. At the 72-hour stage many of the frozen spirochetes exhibited marked granulation, some were straightened and thin with large, external-appearing granules, and others were regularly spiraled. The forms in the control appeared finely stippled or evenly stained. The frozen samples from the 96-hour stage showed many thin, straightened, heavily granulated forms. There were both large and small granules in some of the long organisms. However, some spirochetes had withstood the treatment and appeared to have normal structure. There seemed to be a considerable decrease in the number of organisms present following freezing. The 120-hour specimens, which had been rapidly frozen and thawed, were heavily granulated, straightened, and shrunken; a regularly spiraled, finely granular structure predominated.

The suspensions of *S. novyi* were repeatedly frozen and thawed. A rat was bled 72 hours after inoculation; the blood was defibrinated; and control films were made on one-half of each of 20 slides. The remainder of the blood was frozen in an alcohol, solid carbon dioxide⁴ bath at about -48 C. After 2 minutes the blood was removed and thawed quickly in a water bath at 37 C, and films were prepared on the second halves of four of the slides containing the controls of organisms which had not been frozen. The remaining blood was refrozen, thawed, and four more films made. This was repeated five times. Three of the four slides of each series were stained with Romanowsky's stain and the fourth with Loeffler's flagella stain. No significant differences in the structure of the spirochetes were observed when similar slides employing each stain were compared.

The results of this experiment indicated that there was considerable variation among the organisms of any particular sample. A progressive granulation and swelling resulted in marked fragmentation of the organisms after the fourth and fifth freezing and thawing. Some of the spirochetes became highly granular, shrunken, and misshapen after one freezing and thawing, whereas others withstood the process with no apparent change. The second freezing and thawing produced increased granulation; more of the forms were shrunken and straightened and had large swellings distributed along the cell. The organisms which had been frozen three times showed marked changes in structure. The granules and swellings were bigger than in the earlier samples, and the body of the organisms were so shrunken that the granules seemed to be external. After the fourth freezing, there was an obvious decrease in the number of organisms present. Swelling and granulation had progressed until each cell contained three or four large granules. There was considerable fragmentation, and many of the spirochetes were very misshapen and straightened. Following the fifth freezing, the organisms were fragmented, granulated, and shrunken.

In order to study the influence of variations in temperature on the motility of *S. novyi*, a simple warming and cooling stage was constructed. It consisted of a brass rectangle with one side divided to form two prongs, one of which was

⁴ Commercial dry ice.

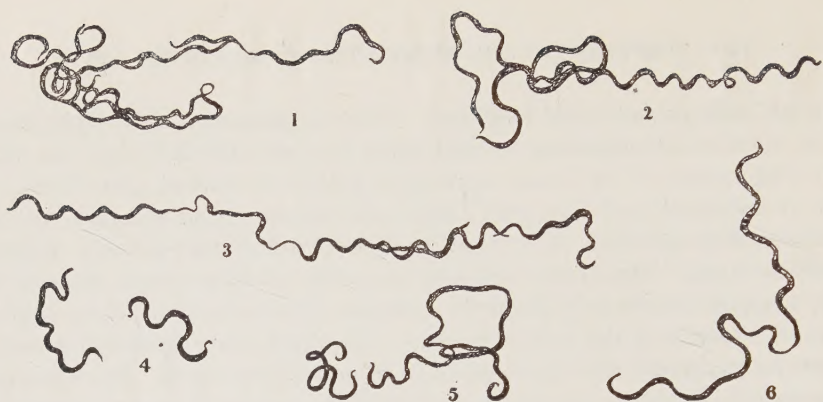


FIG. 1, NOS. 1 TO 6. DRAWINGS OF SPIROCHAETA NOVYI FROM RAT BLOOD FILMS ILLUSTRATING THE NORMAL VARIATION IN THE STRUCTURE OF THE SPIROCHETES

Long coiled forms, organisms in the process of division, and small cells may be found in the same sample. Granulation does not normally progress beyond that shown in no. 6. (Romanowsky's stain, $\times 1500$)

NOS. 7 TO 18. SPIROCHAETA NOVYI FROM RAT BLOOD FILMS PREPARED FROM SPECIMENS FROZEN AND THAWED TWICE

The drawings show the marked granulation resulting from freezing and thawing and the accompanying shrinking and straightening of the spirochetes. The structure of two small forms which withstood the treatment are seen in nos. 10 and 12. (Romanowsky's stain, $\times 1500$)

bent at right angles to permit it to dip into an alcohol-CO₂ bath, the other extended horizontally from the microscope stage to allow a microburner to be placed beneath it. A one-centimeter hole near the center of the stage was placed over the condenser lens of the microscope and a small thermometer attached near the hole by means of silver amalgam. The microscope was placed in a box having sides which projected five centimeters above the stage, a glass window in front of the mirror, and a slit above the window to permit the horizontal prong of the stage to project out over the microburner. By the immersion of the vertical prong in an alcohol-CO₂ bath and the placing of solid CO₂ at the base of the microscope, and then the removal of the CO₂ and cooling bath and the application of heat to the horizontal prong, a variation in temperature of from below 0 C to 37 C could be obtained.

Suspensions of the organisms to be observed were mounted between two cover glasses and placed over the hole in the stage. A film of immersion oil prevented the cover glasses from pulling away from the stage when the objective was raised. A few red blood cells were mixed with the serum suspensions for the following reasons: (1) They aided in focusing the microscope. (2) Slight pressure on the cover glass produced a flow of the cells and could be used as an indication that the medium was fluid. In the later stages of the disease the organisms clumped and adhered to the glass to such an extent that their degree of movement was not a reliable index of the fluidity of the medium. (3) Crenation of the red cells occurred on freezing. Since freezing did not take place simultaneously over the entire film, areas of crenation indicated where freezing had occurred.

The motility of *S. novyi* suspended in blood serum and subjected to changes in temperature at the 48-, 72-, 96-, and 120-hour stages in the disease was observed. The organisms were warmed to 37 C, cooled to freezing, frozen, and thawed. The entire process was limited to approximately one hour in order to minimize the changes in motility as the result of standing. Ten rats were infected and bled at the desired period. The blood was defibrinated and a serum suspension of the organisms obtained by centrifugation of the blood at 1,700 rpm for 10 minutes. A small drop of the suspension was removed from the white fluffy layer at the interface between the cells and serum, mounted between two cover glasses, and examined microscopically. The first observations were made at 25 C, after which the stage was warmed to 37 C and the second observations made. At this time the burner was removed, solid CO₂ placed at the base of the microscope, the vertical prong of the stage immersed in an alcohol-CO₂ bath, and a piece of solid CO₂ put on the warm prong. This lowered the temperature approximately 1 C per minute. Observations were recorded at intervals of 5 to 10 C from 37 C to 0 C. This was repeated for each sample.

It was found that the motion of the spirochetes could be classified as rapid flexion, slow flexion, rapid rotation, slow rotation, very slow rotation, and motionless. During observations, an attempt was made to estimate the relative proportions of organisms exhibiting each of these types of activity. Although there was found to be a wide variation among the organisms of any one prepa-

ration, by estimating the proportion of the organisms exhibiting the different types of motion, a comparison was made of the general trend of the activity as the disease progressed. The results are presented in figure 2.

The organisms appeared to be of two general types: semirigid and flexible. Many of the forms were able to change from one type to the other as the temperature of the medium varied. Most of the extremely flexible types, when cooled sufficiently, became semirigid, evenly spiraled, and took on a rotating motion. Further cooling decreased the speed of rotation or lengthened the pause between reversals in direction of rotation. Upon being warmed, these forms again resumed their original rapid, random, flexion motion. Long organisms showed a tendency to lose the flexion motion earlier in the cooling process than did the shorter ones. Long forms that did not take on the rotating motion upon cooling but continued an irregular flexion appeared to lack

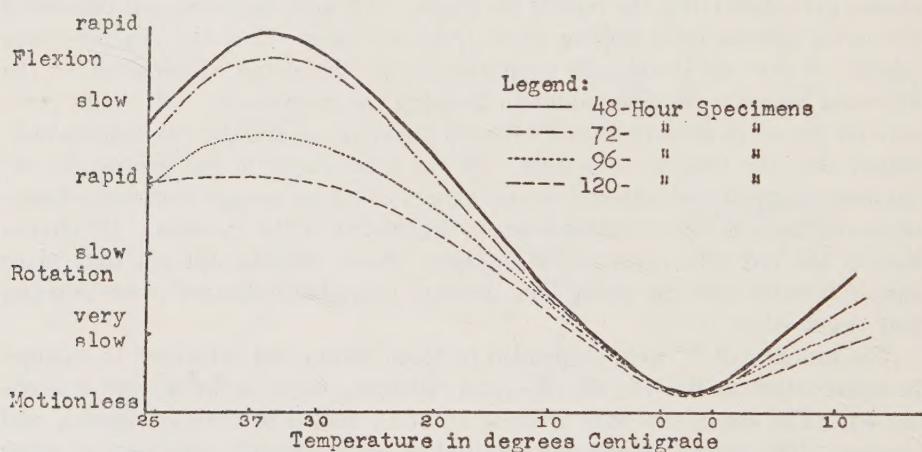


FIG. 2. THE RESULTS OF WARMING AND COOLING ON THE MOTILITY OF RAT BLOOD SERUM SUSPENSIONS OF SPIROCHAETA NOVI PREPARED AT THE 48-, 72-, 96-, AND 120-HOUR STAGES OF THE INFECTION

co-ordination; the ends appeared to act independently. The activity of these organisms decreased with further cooling and stopped earlier in the cooling process than those with a regular rotation. In several instances, however, the flexion gradually changed to a modified irregular rotary motion with each end acting to impede the motion of the other. Some few organisms seemed to rotate slowly in one direction without pause or reversal. Granulation progressed as the cooling continued, but no increase was apparent as the result of freezing. There was no close correlation between granulation and motility. Many of the forms which became motionless early in the cooling process were only faintly granular, and some which remained motile almost to freezing were heavily granular. However, an apparently larger proportion of the bizarre types remained nonmotile on subsequent warming. The spirochetes required 2 or 3 minutes, with the accompanying 3- or 4-degree rise in temperature, before resuming their motion. The first activity was a slow bending which gradually

became rotary, and, with increase in temperature, there was an increase in the rate of rotation. In very few cases was flexion resumed. In general, the motion was more erratic and jerky than that of the control suspensions.

It was observed in some instances that freezing had not occurred when the thermometer registered -5°C , indicating a considerable lag in the cooling process. To correct this, at least in part, the hole in the warming and cooling stage was filled with brass and 5 small holes drilled in the plug. After this modification of the apparatus freezing occurred between 0 and -1°C .

The effects of temperature change and repeated freezing and thawing on the motility of blood serum suspensions of *S. novyi* from a second series of ten rats at 48-, 72-, 96-, and 120-hour stages in the disease, were noted. Using the same technique as in the previous series, the suspensions were warmed to 37°C ,

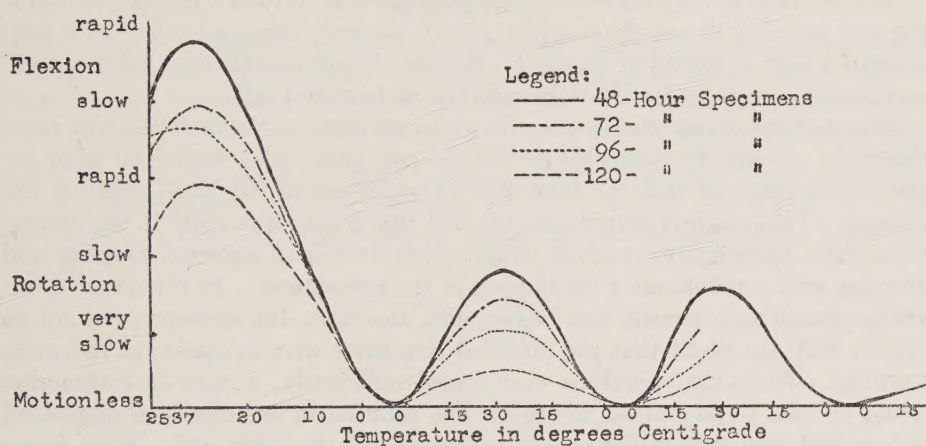


FIG. 3. THE RESULTS OF REPEATED WARMING AND COOLING ON THE MOTILITY OF RAT BLOOD SERUM SUSPENSIONS OF *SPIROCHAETA NOVYI* PREPARED AT THE 48-, 72-, 96-, AND 120-HOUR STAGES OF THE INFECTION

cooled, frozen, thawed, and then warmed to 25°C , re-cooled, frozen, and again thawed and warmed to 25°C . This was repeated three times or until motility had ceased in most of the organisms. Observations were made at intervals of 5°C throughout the entire process. The rate of temperature change in this series was between 1 and 1.4°C per minute.

The results of repeated freezing and thawing on the activity of the spirochetes were similar to those recorded in the first series. Those organisms present in suspensions of serum from rats at the onset of the disease showed greater activity and more successfully withstood repeated freezing and thawing than those in later periods. The results are presented in figure 3.

DISCUSSION AND CONCLUSIONS

Observations of the structure of *Spirochaeta novyi* showed that normally the organisms appeared uniformly stained. When the organisms were cooled, the colloidal structure of the protoplasm seemed to break down sufficiently to form microscopic units. This effect may have been due to the physical action on

the stability of the system or to the changes accompanying the aging process, and, in the later stages of the disease, as a result of the immunological factors in the blood. The entire length of the organism became stippled in appearance. As the process continued, this changed to obvious granulation. In a few instances, granules appeared in but one end of a long form. The process regularly continued until the organism exhibited a distinctly beaded appearance. When spirochetes were subjected to freezing and thawing, the flocculation continued until there were two or three large particles. Coincidentally, there was a marked shrinking and straightening of the spirochetes. The difference in extent of flocculation might be explained by a more complete breakdown of the colloidal system by freezing and thawing than resulted in the ordinary deterioration upon standing.

The decrease in the number of organisms noted after each freezing and thawing was probably due to the fragmentation of severely damaged cells which then appeared only as debris in the field. Repeated freezing and thawing is a well-recognized procedure for the disintegration of bacterial cells.

The motility of spirochetes from blood drawn early in the infection was more decidedly affected by temperature change and more often recovered after repeated freezing and thawing than that of organisms in the later stages of the disease. This was probably due to the fact that most forms early in the disease were short (presumably young) forms which withstood repeated freezing and thawing with a minimum of breakdown of the protoplasm. In the larger spirochetes flocculation already had begun, and, therefore, the systems were not so stable, with the result that the modified structures were incapable of resuming motility. When the organisms were cooled sufficiently, a wave of contraction could be seen to pass along the cell. The rotation in one direction originated at one end, and that of the opposite direction, at the other end. Some forms seemed to rotate continuously in one direction. Apparently the impulse for rotation continued after the organisms became rigid and motionless during the freezing process. The first activity resumed, upon subsequent warming, was a slow flexion, as if the conditions in one region of the cell were suitable for motility. Some shrunken, highly granular forms maintained an erratic motion, indicating the presence of an impulse for motility. The loss of motility of spirochetes when subjected to low temperature might have resulted from either or both of the two following changes: (1) A modification in the protoplasm would have made the organisms rigid and, hence, incapable of motility. Organisms cooled almost to freezing exhibited such rigidity. (2) The destruction of the integrity of the impulse-initiating region in one end would have caused the spirochete to rotate in one direction only, or a modification in both ends would have made it nonmotile.

SUMMARY

When suspensions of *Spirochaeta novyi*, the American strain of the agents of relapsing fever, were subjected to repeated freezing and thawing, a progressive flocculation of the protoplasm resulted in the formation of three or four large

particles with an accompanying shrinking and straightening of the cell. The protoplasm of the organisms consisted of a pale-staining peripheral layer and a dark-staining endoplasm. Normally the latter gave the spirochetes an evenly stained appearance. However, the frozen and thawed specimens exhibited large, dark-staining granules along the thin, pale-staining organisms.

The motility of spirochetes from blood drawn early in the infection was more often regained after freezing and thawing than was that of spirochetes from blood drawn at later stages in the disease. Two general types of organisms were observed: flexible and semirigid. Upon being cooled, the flexible forms took on the rotation characteristic of the long, semirigid organisms, but, with subsequent warming, flexion was restored. Semirigid forms merely increased the speed of rotation with a rise in temperature.

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